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The Role of Monoclonal Antibody Affinity in Tumor Immunotherapy Evaluated in In Vivo Models for Minimal Residual Disease

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Summary: To evaluate the role of affinity in monoclonal antibody (mAb)–mediated treatment of carcinomas, we compared the antibodies 17-1A and 323/A3 that bind with different affinities overlapping epitopes on the epithelial adhesion molecule Ep-CAM. This comparison was performed in several models for minimal residual disease in mice grafted with Ep-CAM transfected B16 melanoma cells originating from C57BL/6 mice. These cells were either grafted subcutaneously or injected intravenously into nude BALB/c mice, or grafted subcutaneously in immunocompetent C57BL/6 mice. In the BALB/c subcutaneous model, significant therapeutic results ($p < 0.05$) compared with the control mAb were obtained with both mAbs 17-1A and 323/A3. However, when treating lung metastases in nude BALB/c mice that had developed after intravenous injection of the B16/Ep-CAM tumor cells, only the high-affinity 323/A3 mAb could significantly ($p < 0.05$) reduce the number of metastases that appeared. In syngeneic C57BL/6 mice grafted subcutaneously with B16/Ep-CAM cells, a single 323/A3 or 17-1A mAb injection had no effect, in contrast to that observed for the nude BALB/c mouse model. However, multiple injections of the 323/A3 mAb significantly ($p < 0.005$) reduced the mean tumor volume, although they did not prevent tumor development. The results show that in vivo antibody-mediated effector cell activation and subsequent tumor cell elimination is determined by mAb affinity and target antigen density. Therefore, treatment of minimal residual disease with high-affinity mAb 323/A3 is expected to improve the clinical results obtained with mAb 17-1A. **Key Words:** Monoclonal antibody—Murine models—NK cells—Affinity.

After a decade of monoclonal antibody (mAb) therapy against solid tumors, it can be concluded that unconjugated mAbs in general lack the efficacy to eradicate large tumor masses. Despite a certain ability to localize into the tumor, and to bind to tumor cells, several factors such as the size and the degree of vascularization of the tumor limit the access of the mAb to the target cells and therefore affect the cytotoxic potential of mAbs in vivo (1,2). However, there could be an important role for mAbs as an adjuvant treatment of patients after resection of the primary tumor. The use of mAbs in eliminating single tumor cells and small tumor nodules that may still be present after tumor resection

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may be useful in prevention of tumor recurrence and could offer a more specific therapy with reduced side effects, compared with the currently used radiotherapy and chemotherapy. As reported by Riethmüller et al. (3), who used murine mAb 17-1A to treat patients with resected Dukes C colorectal carcinoma (3), a 30% increase in 5-year survival of treated patients was observed, largely due to a reduction of the occurrence of distant metastases. The results were at least as good as those obtained using current chemotherapy schemes (4,5), and mAb treatment had significantly lower side effects.

In search of more potent mAbs for immunotherapy of carcinomas, we established that another anti-Ep-CAM mAb, 323/A3, which competes with 17-1A and has a 40-fold higher affinity, is 100–1,000 times more effective against Ep-CAM-positive tumor cells in in vitro antibody-dependent cytotoxicity (ADCC) and complement mediated cytotoxicity (CMC) assays (6). In the current study, we analyzed different murine in vivo models to compare the efficacies of these mAbs for adjuvant therapy of minimal residual disease on either subcutaneously (s.c.) growing tumors or on lung metastases developing after an intravenous (i.v.) injection of tumor cells. Immunologically deficient BALB/c nude mice as well as fully immunocompetent C57BL/6 animals were studied. These models provide an opportunity to evaluate the effect of mAb affinity and of different treatment regimens to optimize treatment of minimal residual disease. In all models, treatment with mAb 323/A3 was found to be more effective than the treatment with mAb 17-1A, but especially when tumors were treated in syngeneic immunocompetent mice. This indicates that for future patient treatment, mAb 323/A3 may also be more advantageous, as could have been concluded from studies in the nude mouse model.

MATERIALS AND METHODS

Cell Lines

Murine B16 melanoma cells derived from C57BL/6 mice were obtained from the ATCC. B16C215 cells clone D2, B16 cells transfected with the Ep-CAM (also known as GA733-2 or C215) cDNA have been described previously (7).

Mice

Six-week-old male BALB/c nu/nu or immunocompetent C57BL/6 SPF mice were obtained from IFFA Credo (St. Germain-sur-l'Arbresle, France). The nude mice were kept in the animal facilities of the Academic Hospital Leiden in sterilized cages under sterile filter top conditions and were handled in a laminar airflow. Sterile food pellets and acidified sterile water were supplied ad libitum. Immunocompetent animals were kept under standard conditions.

Antibodies

The murine IgG2a mAbs 17-1A (8) and 323/A3, a switch variant of the original IgG1 323/A3 mAb (9), directed against the human epithelial cell adhesion molecule Ep-CAM (10,11) were used. MAb 323/A3 ($K_a = 2 \times 10^9\ M^{-1}$) has an ~40-fold higher affinity for Ep-CAM than mAb 17-1A ($K_a = 5 \times 10^7\ M^{-1}$) and can prevent mAb 17-1A from binding to Ep-CAM (12,13). MAb 175F4 a murine IgG2a directed against the MAM-7 antigen, expressed on human renal and breast carcinomas, but not on murine B16 or B16C215 cells, served as a control mAb. The antibodies were purified by protein A sepharose and ion-exchange chromatography on high-performance liquid chromatography (Pharmacia Biotech B.V., Woerden, The Netherlands) and were negative for endotoxin activity by LAL tests.

In Vivo Tumor Models

In the s.c. models, 2.5×10^5 murine B16C215 cells were injected in the right flanks of the mice on day 0. In some experiments, control B16 cells were injected in parallel to B16C215 cells into the left flanks of the mice. In the Winn-type tumor neutralization assay, 1×10^6 tumor cells were incubated with 1 mg/ml mAb in 100 μl phosphate-buffered saline (PBS) for 1 h at 37°C. These cells were then injected s.c. in the presence of the mAbs. In the other s.c. models, mAb therapy started 1 day after grafting with an intraperitoneal (i.p.) injection of 40 or 400 μg mAb. In the multiple injection protocols, 100 μg mAb was injected i.p. starting on day 1, and additional 100 μg injections were given on days 4, 8, 11, and 15. Tumor appearance was registered every other day and tumor sizes were measured twice a week.

In the lung metastasis model, 1×10^5 or 2.5×10^5 B16C215 cells were injected i.v. in the tail vein of nude BALB/c mice on day 0. On day 1, the mice received one i.p. injection with either 40 or 400 μg mAb, respectively. On day 21, mice were killed and

the tumors that were visible on the lung surface were counted. All statistical analyses were performed with the nonparametric Mann–Whitney test.

Flow Cytometric Analysis

To detect binding of murine mAbs 175F4, 17-1A, and 323/A3 to B16 or Ep-CAM transfected B16C215 tumor cells, 1×10^6 cells were incubated in 200 μl PBS + 0.5 bovine serum albumin (BSA) for 1 h at 4°C with 10 μg/ml mAb. Cells were subsequently washed three times with PBS/BSA and incubated for 1 h at 4°C with 100 μl fluorescein isothiocyanate labeled rat anti-mouse IgG (Southern Biotechnology Association, Birmingham, AL, U.S.A.) diluted 1:100 in PBS/BSA. After washing three times with PBS/BSA, cells were resuspended in 300 μl of PBS/BSA containing 1 μg/ml propidium iodide to discriminate between viable and dead cells. From each sample, 10,000 viable cells were examined.

To detect Ep-CAM expression on tumors after treatment with various mAbs, the tumor cells from mAb-treated mice were isolated by squeezing the resected tumor through a metal grid. The isolated tumor cells were subsequently cultured for 4 days, after which all adherent cells were phenotypically identified to the original B16C215 cells. Ep-CAM expression was determined using mAb 323/A3.

Cytotoxicity Assays

ADCC and CMC with murine splenocytes or serum, respectively, were performed as previously described (6). Splenocytes were obtained from a C57BL/6 mouse and were cultured for 3 days in the presence of 200 U of interleukin-2 (Cetus, Amsterdam, The Netherlands), 100 U/ml of murine granulocyte-macrophage colony-stimulating factor (R&D), and 20 μ*M* β2-mercaptoethanol.

RESULTS

In Vitro Sensitivity of the Ep-CAM Transfected B16 Tumor Cells to the mAb-Mediated Effector Mechanisms

The results presented in this article concerning experimental in vivo therapy for minimal residual disease were obtained using the C57BL/6-derived murine melanoma cell line B16 and its Ep-CAM transfectant B16C215. These cells were selected as targets because in nude BALB/c mice, these murine cells are probably less immunogenic than human xenografts, whereas they also permitted studies in immunocompetent C57BL/6 mice. In addition, these cells allow a comparison of two tumors from one cell line either with or without Ep-CAM expression. The treatment of parental B16 cells without Ep-CAM expression served as a negative control.

The antibodies tested were 323/A3 and 17-1A, both reactive with the Ep-CAM molecule, but with different affinity. To investigate whether they recognize closely located epitopes on Ep-CAM, a competition assay was performed in which both mAbs had to compete the 323A3 Fab fragments for binding to a solid-phase adsorbed pure Ep-CAM. As shown in Fig. 1, the 17-1A mAb competes with 323/A3 Fab fragments, although less effectively than the mAb 323A3 itself, which approximately corresponds to the previously reported differences (40–100 times) in the affinities of these mAbs (6).

To confirm that mAb 175F4 is an appropriate control mAb, and to evaluate the binding of 17-1A and 323/A3 to the target cells, the binding to B16 and B16C215 cells was analyzed by flow cytometry. All mAbs tested did not bind to B16 cells (data not shown), whereas 17-1A and 323/A3 both bound to B16C215 cells. However, the high-affinity mAb 323/A3 bound better to B16C215 cells than did the low-affinity mAb 17-1A (Fig. 2A).

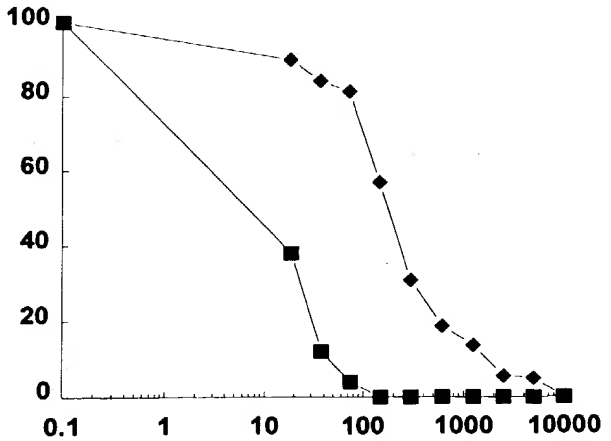

FIG. 1. A competition assay. In a 96-well enzyme-linked immunosorbent assay plate, 500 ng of either monoclonal antibody (mAb) 323/A3 (◆; an IgG2a variant) or mAb 17-1A (■; IgG2A) was tested for binding to a solid-phase adsorbed Ep-CAM in the presence of the 323/A3 Fab fragments (0–10,000 ng/well, *x* axis). The bound mAb was detected using anti-IgG2A-HPRO conjugate. The results are presented as a percent to mAb binding in the absence of the competing Fab fragments (*y* axis).

Using the Ep-CAM transfected B16 tumor cells, two potential effector mechanisms that might be activated by mAbs in vivo were investigated for their efficacy in vitro. ADCC with highly activated C57BL/6 splenocytes was performed with 175F4, 323/A3, and 17-1A mAbs against B16 and B16C215 cells at effector–target ratios (E/T) of 25, 50, and 100:1. The results showed that both mAbs 323/A3 and 17-1A could trigger murine splenocytes to kill Ep-CAM-positive tumor cells, although the percentage lysis obtained with mAb 17-1A was lower than with 323/A3. No ADCC was obtained at E/T 25:1, whereas ADCC levels at E/T ratios 50:1 and 100:1 were comparable, indicating a lysis plateau was reached at E/T 50:1 for this concentration of the mAb. The control mAb 175F4 was unable to mediate ADCC of the B16C215 cells (Fig. 2B). The difference in ADCC between mAbs 17-1A and 323/A3 most likely reflects the differences in binding capacity of these mAbs to the B16C215 target cells. Another mechanism through which antibodies can destroy tumor cells is by activation of the classic complement pathway. CMC experiments were performed on 323/A3 mAb-coated B16C215 and B16 cells with either murine or baby rabbit complement. No complement-mediated lysis of murine tumor cells was obtained in the presence of murine serum, whereas the control baby rabbit complement lysed the cells relatively efficiently (Table 1). The activity of the murine complement was confirmed by the lysis of antibody-coated sheep red blood cells. With baby rabbit complement, mAb 323/A3 could medi-

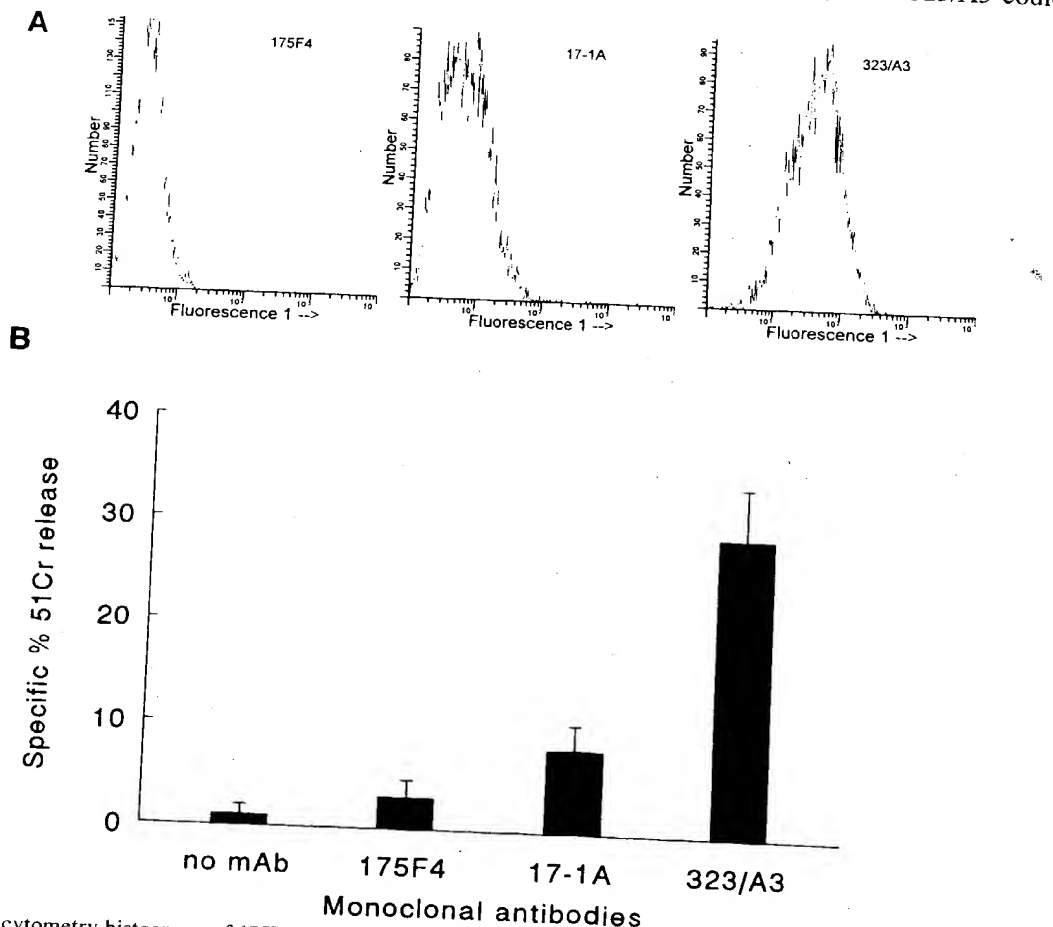

FIG. 2. A: Flow cytometry histograms of 175F4 (negative control), 17-1A, and 323/A3 mAb binding to B16C215 Ep-CAM transfected murine melanoma cells. B: Antibody-dependent cellular cytotoxicity with granulocyte-macrophage colony-stimulating factor plus interleukin-2 preactivated murine splenocytes and murine IgG2a monoclonal antibodies (mAbs) 175F4 (control), 17-1A, and 323/A3 against B16C215 cells. The percentage lysis is represented on the *y* axis by the specific ^{51}Cr release after 18 h from B16C215 cells, mediated by 10 μg/ml mAb at an effector–target ratio of 50:1. The values shown are means of triplicate samples ± SD.

TABLE 1. *Complement lysis assay with murine serum and baby rabbit complement 1:5 diluted with DMEM against B16 and B16C215 cells at different 323/A3 monoclonal antibody concentrations (in micrograms per milliliter); lysis of sheep red blood cells was performed to confirm the complement activity*

	Murine serum			Baby rabbit complement		
B16	2	1	1	1	2	2
B16C215	3	2	1	64	45	12
Sheep red blood cells	89	90	6	92	94	7
Antibody concentration	2	0.2	0	2	0.2	0

ate lysis of B16C215 cells but not of B16 parental cells, indicating that the observed lysis was due to binding of 323/A3 and that the amount of the 323/A3 mAb bound to B16C215 cells was adequate to activate the complement system. The combined in vitro results suggest that in vivo, ADCC may be a more important mechanism of mAb-mediated kill of B16C215 tumor cells than CMC is.

Treatment of Subcutaneous Tumors in BALB/c Nude Mice

To compare the efficacies of mAbs 17-1A and 323/A3 for in vivo tumor cell eradication, BALB/c nude mice were injected s.c. on day 0 with 2.5×10^5 murine B16C215 cells into the right flank and with 2.5×10^5 B16 parental cells into the left flank, followed by a single i.p. injection with either 40 or 400 μg mAb 24 h after tumor cell inoculation. Within 10 days, all mice simultaneously developed a B16 tumor on the left flank, indicating that the mAb treatment did not influence the outgrowth of Ep-CAM negative tumor cells (data not shown). As depicted in Fig. 3, all mice treated with the control 175F4 mAb also developed a B16C215 tumor within 10 days after tumor cell inoculation. The first measurable B16C215 tumor appearance was not affected by treatment with mAb 17-1A. Treatment of the mice with a single injection of mAb 323/A3, however, postponed first tumor appearance until day 9 with the 40 μg injection (Fig. 3A) and until day 11 with injection of 400 μg mAb (Fig. 3B). Although 17-1A did not delay the initial outgrowth of tumors in those mice that did come down with tumor, treatment of the mice with one single injection of mAb 17-1A kept 50% of the mice tumor free, whereas 323/A3 kept 50–75% of the mice tumor free. There was no significant difference between 17-1A and 323/A3 mAb treatment in this model. None of the tumor-free mice developed tumors during the following 3 months after which the experiments were terminated.

The mean tumor sizes of all treated mice was monitored from day 8 until day 18 after which all mice had to be killed because of large tumor volumes of the B16 tumors. Treatment of B16C215 tumors in mice with mAb 17-1A and 323/A3 resulted in significantly lower group mean B16C215 tumor sizes compared with the control group (Fig. 4). Additionally, the mean tumor sizes in the mice that developed a tumor were also significantly lower in the 17-1A and 323/A3 mAb-treated mice compared with the 175F4-treated mice on the same day (Fig. 4). The difference in mean tumor size on day 18 between animals treated with either mAb 17-1A or mAb 323/A3 was not significant. As expected, the mean volumes of B16 Ep-CAM negative control tumors in the mice treated with mAb 17-1A or 323/A3 were not reduced compared with the B16 mean tumor volumes of the control group treated with mAb 175F4 (data not shown).

Treatment of Lung Micrometastases in BALB/c Nude Mice

To simulate the treatment of minimal residual micrometastatic disease in the lung, 1×10^5 B16C215 tumor cells were injected i.v. in the tail vein on day 0 and groups of 10 mice were treated with one i.p. injection of 40 μg 323/A3, 17-1A, or 175F4 mAb on day 1. After 3 weeks, the number of tumors on the lung surface were counted. Although the number of tumor nodules per lung in the control group varied between 12 and 110, treatment with mAb 323/A3 significantly reduced the average number of lung lesions ($p < 0.05$), whereas 17-1A treatment had no effect (data not shown). In a second experiment, 2.5×10^5 cells were injected i.v. and the mice were treated with 400 μg mAb. The control, but also the specific mAb-treated mice developed a greater number of lung tumors than in the first experiment. However, the results obtained were comparable with the first experiment because 17-1A and 175F4 mAb treatment had no effect, whereas 323/A3 treatment significantly ($p < 0.05$) reduced the number of tumor nodules in the lung compared with 175F4 and 17-1A mAb treatment (Fig. 5).

Winn Type Tumor Neutralization in Immunocompetent Syngeneic C57BL/6 Mice

To study the possibility of the mAb-mediated tumor neutralization in a syngeneic model, a Winn-

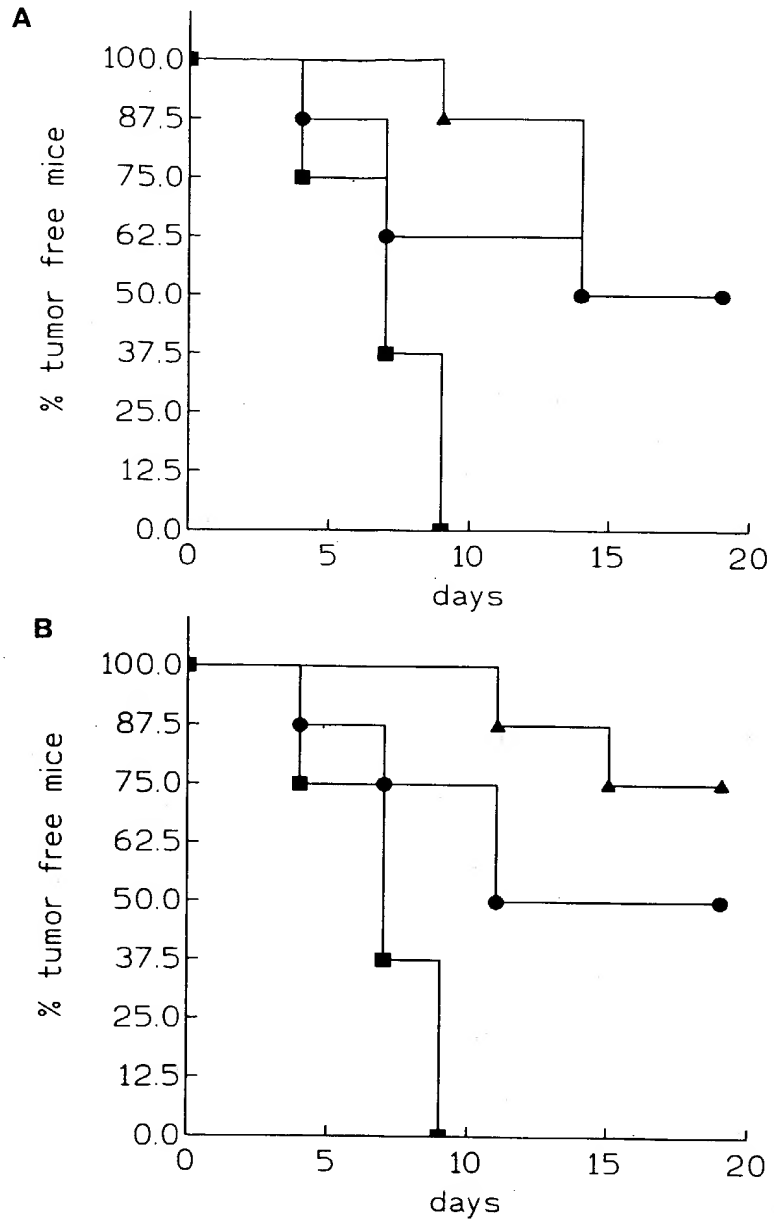

FIG. 3. Tumor appearance in three groups of eight nude BALB/c mice subcutaneously grafted with 2.5×10^5 B16C215 cells on day 0 and treated with one single intraperitoneal monoclonal antibody (mAb) injection on day 1. The percent of tumor-free mice is indicated on the *y* axis and the observation days are indicated on the *x* axis. Mice received either 40 μg (A) or 400 μg (B) of murine IgG2a mAb 175F4 [control (■), mAb 17-1A (●), or mAb 323/A3 (▲)]. Observations of mice for tumor appearance were extended until 3 months after tumor cell grafting and no additional mice developed a tumor during this period.

type assay was performed. Tumor cells were preincubated with antibodies before injection into the mouse, which allows immediate kill of tumor cells by any effector cell present, because all tumor cells are present as single cells with a saturating mAb density on their surfaces. A Winn-type assay in C57BL/6 mice with B16C215 cells preincubated with either mAb 17-1A, 323/A3 or the control mAb 175F4 indicated that both 17-1A and 323/A3 could cause a significant ($p < 0.05$) reduction of the size of the tumors developed (Fig. 6). However, no prevention of tumor outgrowth could be obtained, because all mice developed a B16C215 tumor.

Treatment of s.c. Tumors in Immunocompetent Syngeneic C57BL/6 Mice

The effects of mAb 17-1A and 323/A3 on the growth of grafted mostly single tumor cells was in-

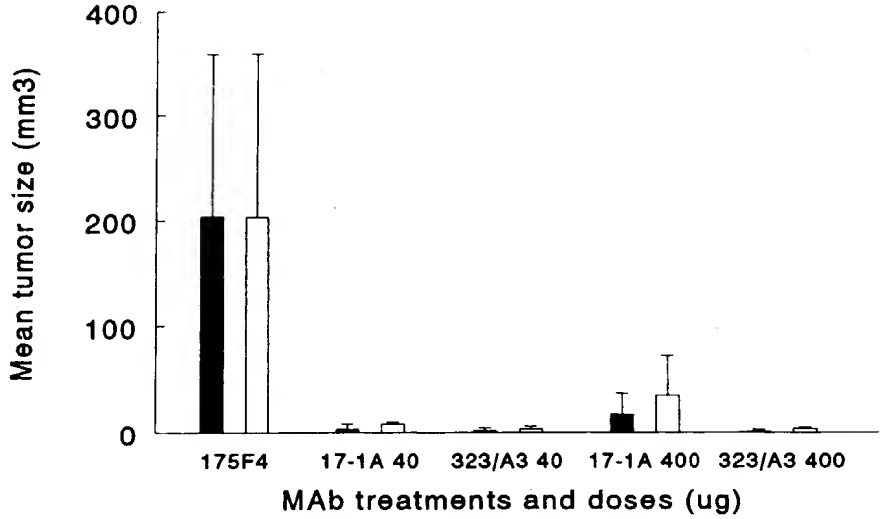

FIG. 4. The mean tumor sizes ± SD on day 18 from five groups of eight mice treated with a single injection of either 40 or 400 μg monoclonal antibody (mAb) 17-1A or 323/A3 1 day after subcutaneous tumor cell grafting (see Fig. 2). Because both 40- and 400-μg doses resulted in similar mean tumor sizes in the 175F4 control mAb-treated groups, only the mean tumor size obtained with the 400-μg 175F4 mAb treatment is presented. Both the group mean (■) and the tumor mean of the mice that developed a tumor (□) are presented. Tumor sizes were measured in three dimensions and are expressed in cubic millimeters as indicated on the *y* axis. The treatment of the group is indicated on the *x* axis.

vestigated in an immunocompetent syngeneic model. Six groups of eight C57BL/6 mice were injected s.c. with 2.5×10^5 B16C215 tumor cells followed after 24 hours by a single i.p. mAb injection of 100 μg 175F4 (control), 17-1A, or 323/A3. Three additional groups received further mAb injections on day 4, 8, 11, and 14. This treatment regimen was based on our previous observations that mAbs after

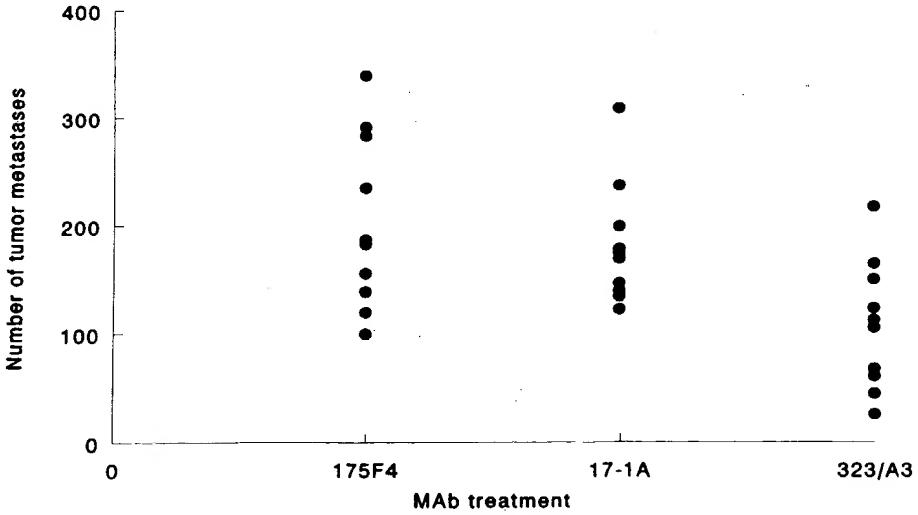

FIG. 5. The number of lung metastases that have developed by day 21 in nude BALB/c mice (three groups, 10 animals each) treated with anti-Ep-CAM or control monoclonal antibody (mAb). The number of metastases is shown for individual mice. On day 0, all mice received an intravenous injection of 2.5×10^5 B16C215 murine melanoma cells followed by an i.p. injection of 400 μg mAb on day 1. The mAb injected into the mice of the respective group (17-1A, 323/A3 or the control 175F4) is indicated on the *x* axis, and the number of lung metastases is indicated on the *y* axis. Only 323/A3 mAb treatment resulted in a significantly lower mean number of lung metastases on day 21 ($p < 0.05$ when the group mean numbers of metastases are compared).

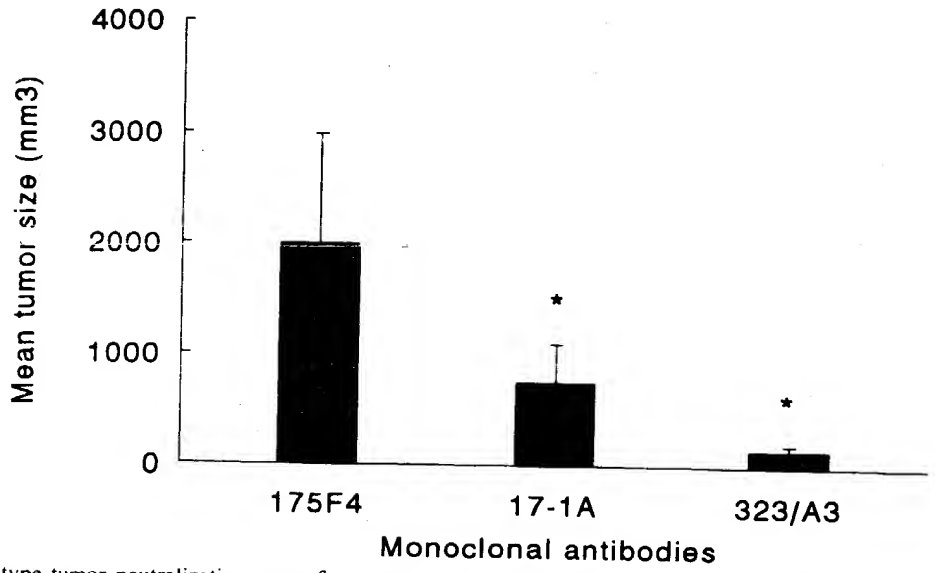

FIG. 6. A Winn-type tumor neutralization assay for treatment of B16C215 cells in immunocompetent C57BL/6 mice. On day 0, B16C215 murine C57BL/6 melanoma cells were incubated before injection with 1 mg/ml of either murine IgG2a 17-1A, 323/A3, or 175F4 (control) monoclonal antibody (mAb) in phosphate-buffered saline at 37°C for 1 h. One hundred microliter mAb solution containing 10^6 cells was subsequently injected s.c. in the flank of three groups of C57BL/6 mice. For every mAb treatment (represented on the *x* axis), the mean tumor sizes ± SD (indicated on the *y* axis) on day 21 are presented in this figure. Mean tumor sizes were measured in three dimensions and are presented in cubic millimeters. Mean tumor sizes that differed significantly ($p < 0.05$) from the mean tumor size in the 175F4-treated group are indicated by asterisks.

i.p. injection were present in the tumor until at least 72 h after injection (2). The first tumors in all groups started to appear at day 9, and within 13 days all mice had developed a solid tumor (data not shown). The mean tumor size in the mice that received a single mAb injection did not differ between the groups (Fig. 7A). However, mice that received multiple 323/A3 mAb injections had significantly ($p < 0.005$) smaller tumors than the 175F4 and 17-1A mAb-treated mice (Fig. 7B). At day 15, the observation of the 175F4- and 17-1A-treated groups had to be terminated because the tumor sizes in most of these mice exceeded the ethically tolerable size. In three separate experiments, only multiple injections but not single injections with mAb 323/A3 resulted in significantly smaller tumors. Of a total of 30 mice that received multiple 323/A3 injections, 3 mice stayed tumor free.

Antigen Expression on Tumor Cells After mAb Treatment

To study the Ep-CAM expression on tumor cells from the tumors that had developed despite the mAb treatment, tumors from C57BL/6 mice from each group were isolated, and the Ep-CAM expression on tumor cells was determined by flow cytometric analysis with mAb 323/A3 (Fig. 8). Ep-CAM expression on tumor cells from mice treated with multiple injections of the 323/A3 mAb was found to be significantly ($p < 0.01$) lower in comparison to the tumor cells from 175F4 mAb-treated mice. Ep-CAM expression on 17-1A- and 175F4-treated tumors was similar to Ep-CAM expression on the cells that were maintained in vitro. These results suggest that the reduced tumor size observed after treatment with 323/A3 was due to the preferential elimination of relatively high Ep-CAM-expressing tumor cells.

DISCUSSION

The efficacy of treating minimal residual disease with mAb 17-1A, a mAb currently approved for adjuvant treatment of patients with resected colon carcinoma stage Dukes C, was compared with the treatment efficacy of mAb 323/A3 in several different in vivo murine models. Both mAbs are directed against the human Ep-CAM molecule (10,11). mAb 323/A3, however, has a 40-fold higher affinity for Ep-CAM than mAb 17-1A does (12,13), and was previously found to be much more effective than

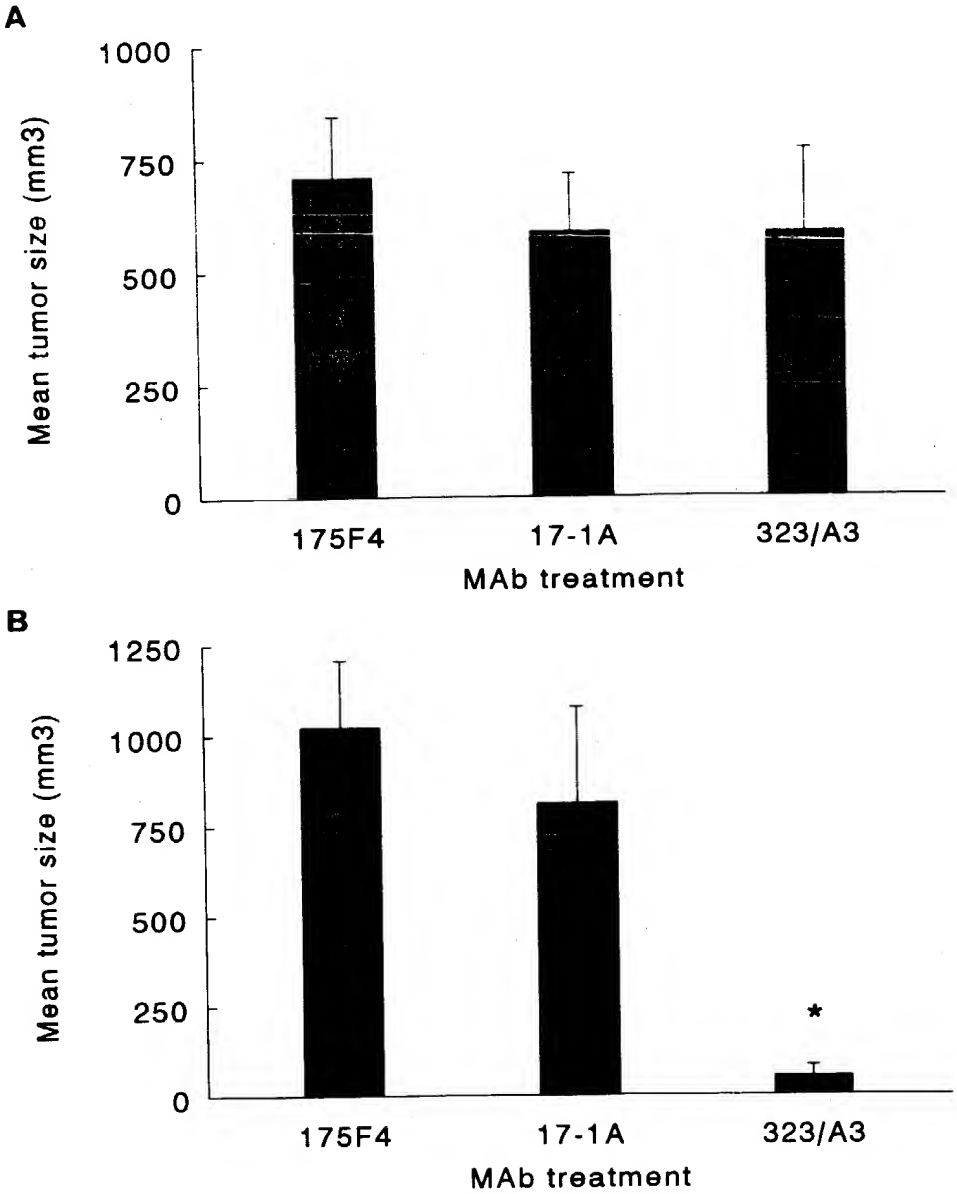

FIG. 7. Mean tumor sizes on day 15 of s.c. C57BL/6 derived B16C215 tumors in groups of eight immunocompetent C57BL/6 mice that received either single (A) or multiple (B) intraperitoneal (i.p.) injections with murine IgG2a monoclonal antibodies (mAbs) 175F4 (control), 17-1A, or 323/A3. The mAb treatment received by the mice is indicated on the x axis and mean tumor sizes ± SD (in cubic millimeters) of the group are indicated on the y axis. Mice received 2.5×10^5 B16C215 Ep-CAM transfected C57BL/6 melanoma cells subcutaneously on day 0, followed by either a single i.p. mAb injection of 100 μg on day 1 (A), or multiple mAb injections on days 1, 4, 8, 11, and 14 (B). The group with mean tumor size that significantly ($p < 0.005$) differed from the mean tumor size in the 175F4 mAb-treated control on day 15 is indicated by an asterisk.

mAb 17-1A in mediating tumor cell kill in vitro by ADCC and CMC (6). We (2) and many others have successfully treated nude mice xenografted with human tumor cells with mAbs against human tumor-associated antigens. In this study, we used a murine melanoma cell line transfected with human Ep-CAM, which offered the opportunity to study tumor treatment in a nude mouse as well as in a syngeneic

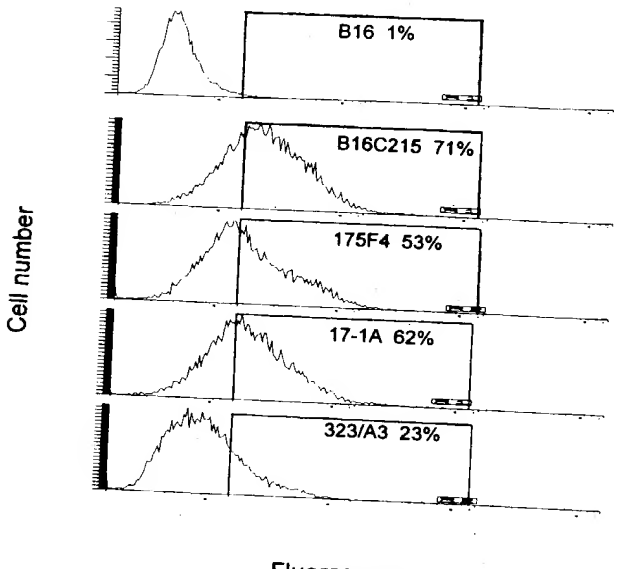

FIG. 8. A representative flow cytometric determination of Ep-CAM expression on B16C215 murine melanoma cells isolated from monoclonal antibody (mAb)-treated mice by murine 323/A3 and a fluorescein isothiocyanate conjugated goat anti-murine IgG mAb. On day 21, five tumors from each group of B16C215 tumor-bearing mice that had received multiple mAb 17-1A, 323/A3 or 175F4 treatment (see Fig. 6B) were isolated, disseminated, and cultured for 4 days in vitro. On day 5 after isolation, the Ep-CAM expression on these cells was determined by flow cytometric analysis. The relative fluorescence intensity that linearly corresponds with the antigen density detected on the cells is indicated by the channel numbers and is represented on the *x* axis. The number of cells per channel is indicated on the *y* axis. Ep-CAM expression was determined on B16 and B16C215 cells that were maintained in vitro and on isolated B16C215 cells from mice treated with mAbs 175F4, 17-1A, and 323/A3, respectively. The percentage of cells that were detected in channel numbers 10–1,000 are indicated in the boxes.

immunocompetent model. Treatment of human Ep-CAM transfected murine tumors in syngeneic mice has the advantage that this model is closer to the situation in patients than human tumor cell xenografts in nude mice, while still permitting the use of mAbs that can be used in patients.

Herlyn and Koprowski showed that complement depletion in mice had no influence on mAb-mediated tumor cell kill even with human xenografts (14). Based on the presence of complement inhibitors on autologous cells, which are generally increased on tumor cells (15,16), it is likely that in vivo complement-mediated cytotoxicity by mAbs hardly contributes to tumor cell kill. Our in vitro CMC results on autologous B16C215 cells with murine complement also suggest that in our treatment models, CMC plays no major role. On the other hand, macrophage and monocyte (14) as well as natural killer (NK) cell depletion (17) in mice was found to significantly reduce mAb-mediated ADCC against tumors. Therefore, ADCC is expected to be the main effector mechanism through which mAbs generate a cytotoxic effect against tumor cells in vivo. Our ADCC results confirmed that ADCC can also act against the Ep-CAM-transfected B16 tumor target cells used in this study. We found that it was necessary to highly activate the NK cells in vitro to obtain ADCC mediated B16C215 tumor cell kill by C57BL/6 splenocytes, in agreement with previous results (17), which indicated a very inefficient ADCC-mediated kill of B16 cells by C57BL/6 effector cells. This most probably is due to the fact that effector NK cells and target cells are syngeneic.

In the model in which BALB/c nude mice were s.c. grafted with B16C215 and B16 cells and subsequently treated with a single mAb injection, both mAb 17-1A and 323/A3 treatments were able to protect at least 50% of the nude mice against tumor formation by B16C215 cells, while not affecting the outgrowth of Ep-CAM-negative B16 tumors. With respect to the number of mice that remained tumor free and the mean tumor sizes, treatment of nude BALB/c mice with mAb 323/A3 and mAb 17-1A were effective ($p < 0.05$) compared with the control 175F4 mAb. Similar effects were observed at both mAb concentrations tested, although treatment with 400 μg mAb 323/A3 could prevent tumor formation in 75% of the mice.

The differences in treatment efficacy between mAb 17-1A and 323/A3 were more evident in the lung model, in which nude BALB/c mice injected i.v. with B16C215 tumor cells were treated with a single intraperitoneal mAb injection. Treatment with mAb 323/A3 in this model was found to significantly reduce the number of lung metastases compared with treatment with mAb 17-1A and to a control mAb. The reason why no mice remained tumor free in this model may be the previously reported (17) low number of Fc receptor-positive effector cells present in the murine lung. Additionally, injection of tumor cells into the tail vein leads to trapping of the cells in the capillary bed of the lung, where the tumor cells can immediately block the blood flow through the vessel, thereby becoming less accessible for mAbs and effector cells than tumor cells injected into the subcutaneous space, and escaping from mAb-mediated kill.

Because nude mice are athymic, their T-cell number is severely decreased; however, they have sev-

eral other possibilities for eradicating tumor cells (18). The percentage of NK cells in peripheral blood and spleens of nude mice is much higher than in euthymic mice, 18.3 versus 2.5%, respectively (19,20). Cytolytic activity of NK cells in nude mice reaches the highest levels between 5 and 8 weeks and is superior to NK activity in euthymic mice (19,20). NK cells are active against a variety of allogeneic tumor cells and are active against major histocompatibility complex (MHC) class I-negative syngeneic tumor cells (21). NK cell activation can be inhibited by a signal transmitted on recognition of self MHC class I (22). Correa et al. showed that antibodies can activate NK cells through their Fc receptors, but that the signal from syngeneic MHC class I-positive target cells can significantly reduce the extent of ADCC (23). They concluded from their experiments that the ADCC level is determined by an interplay between the relative strengths of the activating and inhibitory signals (23). These observations may explain why in our model, mAb 17-1A could prevent tumor outgrowth in the nude BALB/c mice but not in the syngeneic immunocompetent C57BL/6 mice. Treatment of B16C215 tumors in allogeneic BALB/c nude mice, where the NK activation by mAb 323/A3 and 17-1A is not inhibited by syngeneic MHC class I, resulted in an efficient kill of tumor cells. However, in the C57BL/6 mice, the syngeneic MHC class I inhibitory signal may decrease the efficacy of NK cell-mediated ADCC. A high mAb affinity will enhance mAb binding to the tumor cells and therefore give a stronger activation signal to NK cells. This may explain why in our syngeneic C57BL/6 mice, a significant reduction of B16C215 tumor volume was only found after multiple treatments with 323/A3 and not with single treatment or multiple treatments with mAb 17-1A. However, using very high antibody concentrations on single tumor cells, as in the Winn-type assay, mAb 17-1A also may trigger NK cells, leading to lysis of grafted tumor cells, although less efficiently than in BALB/c mice. In BALB/c mice s.c. grafted with human LS180 colorectal carcinoma cells, another allogeneic model studied previously both 17-1A and 323/A3 mAb treatment also resulted in cures in >50% of the mice (2). Therefore, the differences observed between tumor eradication with mAbs in nude BALB/c mice and immunocompetent C57BL/6 mice, respectively, are likely to be dependent on the genetic background of the tumor cells as well as on the total numbers of NK cells and other effector cells.

On the residual tumor cells in five C57BL/6 mice after treatment with mAb 323/A3, Ep-CAM expression was significantly decreased compared with Ep-CAM expression on B16C215 tumor cells treated with mAb 17-1A, 175F4, or in vitro. This observation suggests that only tumor cells with high Ep-CAM expression were killed by the multiple 323/A3 mAb injections. Our results in C57BL/6 mice are in contrast to the observation in nude BALB/c mice by Johansson et al. (1), who found no difference in Ep-CAM expression between tumor cells isolated from mAb-treated mice versus control mice after 10 daily mAb injections with mAb C215, which is also directed against Ep-CAM. In the xenograft model by Johansson et al., NK cells were activated through mAb binding, but did not receive inhibitory signals from self MHC molecules. This may have led to a higher activation of NK cells, and subsequently to a better kill of tumor cells, including low Ep-CAM-expressing cells. In our syngeneic C57BL/6 model, however, only the high-expressing tumor cells may have been able to bind enough high-affinity mAb to overcome the self MHC class I inhibitory signal and could activate the NK cells to kill the tumor cells. Exploiting this hypothesis, only tumor cells with high Ep-CAM expression would be eliminated and the eventually outgrowing tumor will consist of tumor cells with a relatively low Ep-CAM expression level. If correct, this observation supports the selection of high-affinity mAbs for treatment of patients. Because NK cells are important ADCC effectors (17) and negative signals through MHC class I recognition are likely to reduce the efficacy of low-affinity mAbs, it becomes important to use high-affinity mAbs in therapy of patients. In addition, other effector cells such as monocytes, macrophages, and granulocytes may also play a role in mAb treatment of patients.

From the present study, we conclude that tumor therapy with the high-affinity 323/A3 was more effective than treatment with the low-affinity mAb 17-1A. The Winn-type tumor neutralization assay indicated that immediate mAb treatment of single tumor cells is most effective, and that even a 1-day delay of mAb treatment against single tumor cells reduces the final outcome of the treatment. In the syngeneic immunocompetent C57BL/6 mice, mAb-mediated tumor cell elimination was reduced, possibly due to inhibition of NK cell activation by self MHC class I recognition. Only the high-affinity mAb 323/A3 was effective against high antigen-expressing tumor cells. In addition, our in vivo

treatment results with mAbs alone support previous recommendations (17,24) to use high-affinity mAbs in combination with cytokines to improve the results obtained so far in patients with mAb 17-1A (3).

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